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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Rheb (Ras Homolog Enriched in Brain), a member of the Ras family of GTPases, has been implicated as an oncogene and may be involved in estrogen-dependent breast cancer. Rheb activity is induced by non-genomic estrogen signaling; activated Rheb leads to activation of the mTOR kinase and increased protein translation and cell cycle progression. Whether Rheb is required for estrogen-induced breast cancer growth and whether Rheb can promote tamoxifen resistance has not been determined. The purpose of these studies was to determine the contribution of Rheb to the growth and progression of estrogen-dependent and tamoxifen-resistant breast cancers. Thus, I developed short hairpin RNA (shRNA) targeting Rheb1 to reduce its expression in MCF-7 breast cancer cells. Knocking down Rheb1 alone did not significantly affect estrogen-dependent cell proliferation, tamoxifen sensitivity, or anchorage-independent growth. However, it is possible that the closely related isoform Rheb2 may compensate for the loss of Rheb1 activity and thus complicate my results. These results suggest that inhibition of Rheb1 alone may not be beneficial treatments for breast cancer, but it remains possible that pharmacological agents targeting both Rheb1 and Rheb2 could show clinical activity in tumors with elevated Rheb activity.

15. SUBJECT TERMS
Breast cancer, Estrogen Receptor, tamoxifen, small GTPase, Rheb, farnesyltransferase inhibitor

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Table of Contents

INTRODUCTION

My proposed studies focused on two estrogen-regulated Ras family small GTPases, Rheb and Rerg, that have been implicated in breast cancer. Members of the Ras family of small GTPases function as GTP/GDP regulated switches that relay cellular signals involved in cell proliferation, differentiation, and survival. While the Ras oncoprotein is mutationally activated in 30% of all cancers, it is only mutated in about 5% of breast cancers. However, it is now becoming evident that other Ras family GTPases play critical roles in breast cancer biology [1]. The expression or activity of two Ras-related proteins, Rerg and Rheb, is regulated by estrogen, further implicating them in breast cancer tumorigenesis. Rerg (Ras-related, Estrogen Regulated, Growth inhibitor) is a Ras-related candidate tumor suppressor and transcriptional target of the estrogen receptor (ER); its expression is lost in all ER-negative breast cancers. Last year, I reported my findings on Rerg and have since switched my focus to defining the role of Rheb in estrogen-regulated breast cancer.

Estrogen-regulated breast cancers account for two-thirds of all breast cancers. Recently, Rheb1 has been implicated in estrogen-mediated breast cancer growth. Rheb1 is a critical component of the Akt-TSC-mTOR pathway which regulates protein translation, nutrient sensing, cell size, and cell proliferation, and is frequently overexpressed or hyperactivated in human cancers, including breast cancer [1-5]. Furthermore, Rheb1 has been implicated as an oncogene: it is capable of transforming cells [6] and overexpression of Rheb1 can induce tumor formation in mouse models of lymphoma and prostate cancer [7, 8]. Recent studies found that stimulation of ER-positive MCF-7 breast cancer cells with estrogen causes Rheb1 activation, leading to mTOR-mediated phosphorylation of the mTOR substrate p70 S6 kinase [9]. Additionally, Rheb1 was shown to be required for estrogen-induced DNA synthesis and cell cycle progression in MCF-7 cells [9]. The purpose of my studies was to determine the role of Rheb1 in estrogen-induced breast cancer growth its contribution to tamoxifen resistance. Thus, I developed interfering short hairpin RNA (shRNA) to stably repress Rheb1 expression in MCF-7 cells. I found that reducing endogenous Rheb1 expression caused a limited decrease in the anchorage-independent growth of MCF-7 cells, but it did not significantly decrease long-term estrogen-stimulated growth, suggesting that Rheb1 may not be a critical mediator of estrogen-induced breast cancer cell proliferation. Furthermore, reducing Rheb1 expression did not significantly affect tamoxifen sensitivity. One possible explanation for the limited to no role for Rheb1 seen in my studies is that there may be overexpression of the functionally related Rheb2 in breast cancer cells. Another explanation is that full suppression of Rheb1 function is needed to see influences on estrogen and tamoxifen activities. Therefore, my current studies are focused on evaluating pharmacologic methods to inhibit Rheb1 and Rheb2 signaling and function in breast cancer, and thus could have important clinical implications for the treatment of patients bearing tumors with elevated Rheb activity. Additionally, I am determining if Rheb1 and Rheb2 are functionally distinct or identical, to assess the possible role of Rheb2 in compensating for Rheb1 loss.

BODY

Based on revised Statement of Work, submitted and approved in October 2007

Statement of Work Task 1: To determine if loss of Rerg promotes breast cancer tumorigenicity and invasion.

Completed last year and described in my 2007 annual report.

Statement of Work Task 2: To determine the role of Rheb in estrogen-induced breast cancer growth.

To determine if Rheb1 is required for long-term estrogen-induced cell proliferation and for anchorage-independent growth, I developed a retrovirus-based short hairpin interfering RNA (shRNA) targeting Rheb to cause sustained repression of endogenous Rheb1 expression in MCF-7 breast cancer cells. I retrovirally infected MCF-7 cells with vector-based shRNA targeting three different cDNA sequences of Rheb1 as well as with three two controls: empty vector, non-specific shRNA targeting GFP, and non-specific shRNA targeting Luciferase. I confirmed knockdown of Rheb1 using western blot analysis and showed that knocking down Rheb1 decreased phosphorylation of the ribosomal subunit S6, a downstream target of mTOR (Fig. 1A). To determine whether knocking down Rheb1 decreased estrogen-induced cell proliferation, I used the standard MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cell viability assay. Five-day estrogen treatment significantly increased cell viability in control cells (Fig. 1B). Knocking down Rheb did not significantly decrease estrogen-induced cell proliferation. Interestingly, although expression of Rheb1 remained suppressed in Rheb1 knockdown cells following five days of estrogen treatment, phospho-S6 was no longer suppressed (Fig. 1C). These results suggest that other proteins may be compensating for the loss of Rheb1 in these cells. Interestingly, the related Rheb isoform Rheb2 (RhebL1) is highly expressed in MCF-7 cells [5]. It is possible that expression of Rheb2 alone is sufficient to sustain estrogen-induced cell proliferation. Therefore, I plan to knock down Rheb2 together with Rheb in order to ascertain the requirement of Rheb activity in estrogen-induced MCF-7 cell proliferation. I also found that while knocking down Rheb1 may have slightly decreased anchorage-independent growth in soft agar, knocking down Rheb1 did not display a strong effect in this assay (Fig. 1D). Again, knocking down Rheb2 in combination with Rheb1 may more strongly inhibit anchorage-independent growth.

Unexpectedly, I found that long-term estrogen treatment significantly upregulated Rheb1 protein expression (Fig. 1C), uncovering another mechanism by which estrogen activates Rheb signaling, in addition to activation of upstream components of the Rheb pathway. My future studies will determine whether this increase in Rheb1 expression is the result of increased Rheb1 gene transcription or translation.

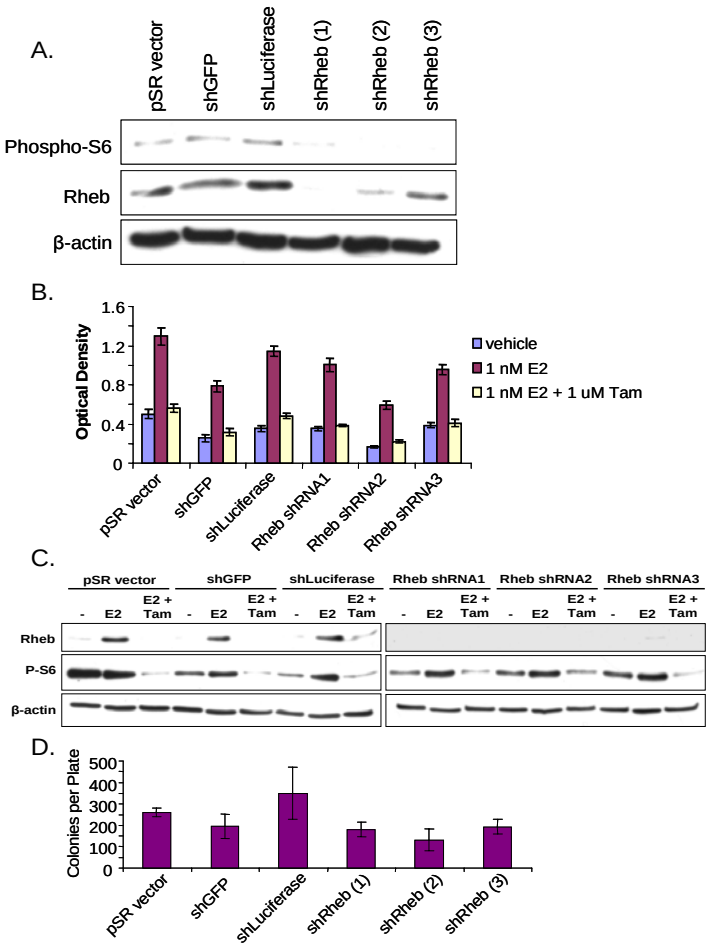

Fig. 1. Knocking down Rheb1 expression does not significantly reduce estrogen-induced MCF-7 cell proliferation or anchorage-independent growth. A. MCF-7 cells were stably infected with empty pSuper.retro.puro vector, or encoding shRNA targeting green fluorescent protein (GFP) or luciferase (negative controls), or Rheb1. Western blots were probed with antibodies to phospho-S6, Rheb1, or β-actin (loading control). **B.** MCF-7 stable cell lines were seeded at equal numbers in 96-well plates and treated with Vehicle, 1 nM estrogen, or 1 nM estrogen + 1 μM tamoxifen for 5 days. Cell viability was measured using the MTT assay. Results represent the average of 8 replicate wells; error bars represent standard deviation. **C.** Western blot analysis of cells treated as described in (B). Cell lysates were probed with antibodies to Rheb1, phospho-S6, or β-actin (loading control). **D.** MCF-7 stable cells were seeded in triplicate into 0.4% soft agar over a 0.6% bottom layer. After 27 days, viable colonies were stained in 2 mg/mL MTT. ImageJ software was used to quantify number of colonies from images of scanned plates. Results represent the average of triplicate plates; error bars represent standard deviation.

I had also proposed to inhibit Rheb1 function in MCF-7 cells by stable transfection of the tumor suppressor TSC2, a GTPase activating protein (GAP) and negative regulator of Rheb1 and Rheb2. TSC2 overexpression would be expected to inhibit Rheb2 in addition to Rheb1. Thus, I attempted to stably transfect MCF-7 cells with ectopic TSC2. During selection, ectopic expression of TSC2 decreased cell proliferation, as expected for a tumor suppressor (Fig. 2). However, while I succeeded in transiently transfecting MCF-7 cells with ectopic TSC2, I was unable to obtain stable colonies overexpressing ectopic TSC2 (Fig. 2). Unfortunately, selective pressure against stable ectopic expression of tumor suppressors (e.g., p53) is a common technical limitation. The development of an inducible TSC2 expression system would allow me to complete the studies proposed in Task 2.

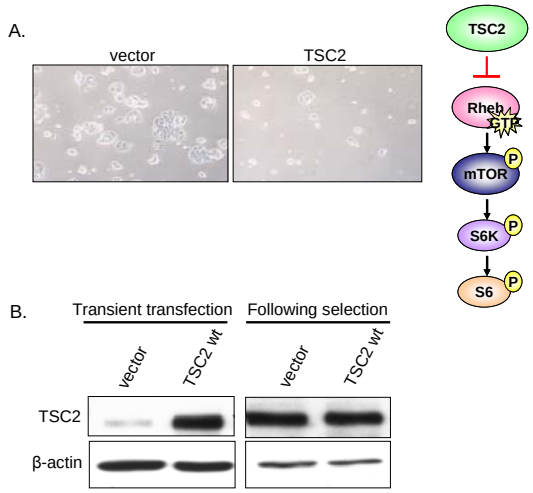

Fig. 2. Stable transfection of the RhebGAP TSC2 in MCF-7 cells. A. MCF-7 cells were transfected with the pcDNA3 empty vector or encoding TSC2. Transfected cells were selected with G418 to establish mass populatios of stably-transfected cells. Surviving colonies were photographed during selection. **B.** MCF-7 cells were transiently transfected with the pcDNA3 empty vector or encoding TSC2, and then selected with G418. Cell lysates were probed using antibodies to TSC2 protein (tuberin) and β-actin (loading control).

I also proposed to determine whether Rheb1 activation promotes estrogen-dependent and -independent growth in breast cancer cells. To this end, I created MCF-7 cell lines stably expressing a constitutively activate Rheb1 construct (Rheb N153T) [10, 11] (Fig. 3A). My preliminary results suggest that activated Rheb1 may increase estrogen-dependent cell proliferation (Fig. 3B). Taken together, my results suggest that while knocking down Rheb1 alone is not sufficient to decrease estrogen-induced cell proliferation (perhaps due to the presence of Rheb2), ectopic expression of activated Rheb may facilitate estrogen induction of cell proliferation.

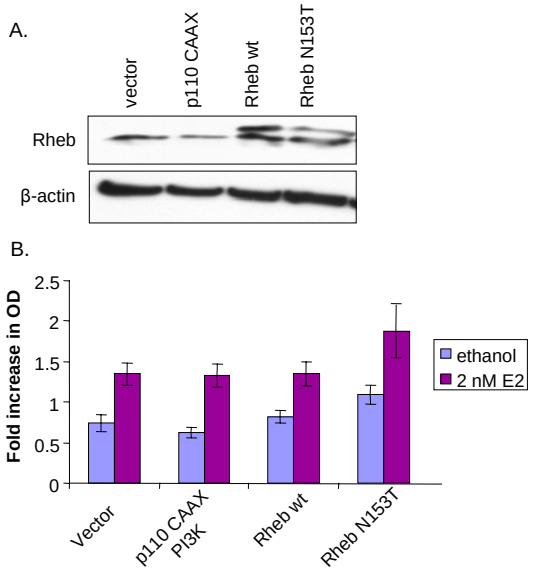

Fig. 3. Stable expression of activated Rheb increases estrogen-induced cell proliferation in MCF-7 cells. A. MCF-7 cells were stably transfected with the pcDNA3 empty vector or with encoding p110 CAAX (an activated version of PI3-kinase catalytic subunit, an upstream activator of Rheb), FLAG-tagged wild-type Rheb1, or FLAG-Rheb1 N153T. Western blots were probed with antibodies to Rheb1 and β-actin (loading control). **B.** MCF-7 stable cell lines were seeded in replicates of 8 in 96-well plates and treated with either ethanol or with 2 nM estrogen for 6 days. Cell viability was measured using the MTT assay. Data represent an average of 8 replicate wells; error bars represent standard deviation.

Statement of Work Task 3: To determine the role of Rheb in the development of tamoxifen resistance.

I have not yet completed most of the experiments proposed in task 3. However, I did find that knocking down Rheb1 did not increase tamoxifen sensitivity in MCF-7 cells (Fig. 1B), suggesting that Rheb1 may not have a vital role in the development of tamoxifen resistance.

Although not proposed in my Statement of Work, my recent work addresses the possibility that Rheb is a clinically relevant target for farnesyltransferase (FTase) inhibitors (FTIs), which are currently in clinical trials for the treatment of breast cancer [12, 13]. Rheb1 is targeted to endomembranes via its carboxy-terminal CAAX tetrapeptide motif, a substrate for posttranslational modification catalyzed by FTase, leading to covalent addition of a farnesyl isoprenoid lipid to the cysteine residue of the CAAX motif. Farnesylation and proper localization are critical for Rheb1 function [14]. FTIs were shown previously to potently inhibit the growth of MCF-7 cells [15], but whether the sensitivity of MCF-7 cells to FTIs is due to Rheb1 inhibition is not known. To determine if the anti-tumor activity of FTIs in breast cancer is due to Rheb1 inhibition or to inhibition of other FTase substrates, I engineered a mutation in the Rheb1 CAAX motif (CSVM to CVLL) that causes it to be modified by the related geranylgeranyltransferase-I (GGTase-I) enzyme rather than by FTase, resulting in a functional Rheb1 protein. I stably transfected MCF-7 cells with this FTI-insensitive Rheb1 variant, geranylgeranylated Rheb1 (GG-Rheb). I found that GG-Rheb1 was resistant to FTI inhibition as measured by the absence of an SDS-PAGE mobility shift (Fig. 4A), but did not reduce the sensitivity of MCF-7 cells to FTI inhibition of cell proliferation (Fig. 4B) or anchorage-independent growth (Fig. 4C). Furthermore, knocking down Rheb1 did not affect the FTI sensitivity of MCF-7 cells (Fig. 4D). These studies suggest that Rheb1 is not the critical target of FTI-mediated growth inhibition in breast cancer. My future work will address whether other pharmacological agents that interfere with Rheb posttranslational processing can inhibit Rheb function in breast cancer.

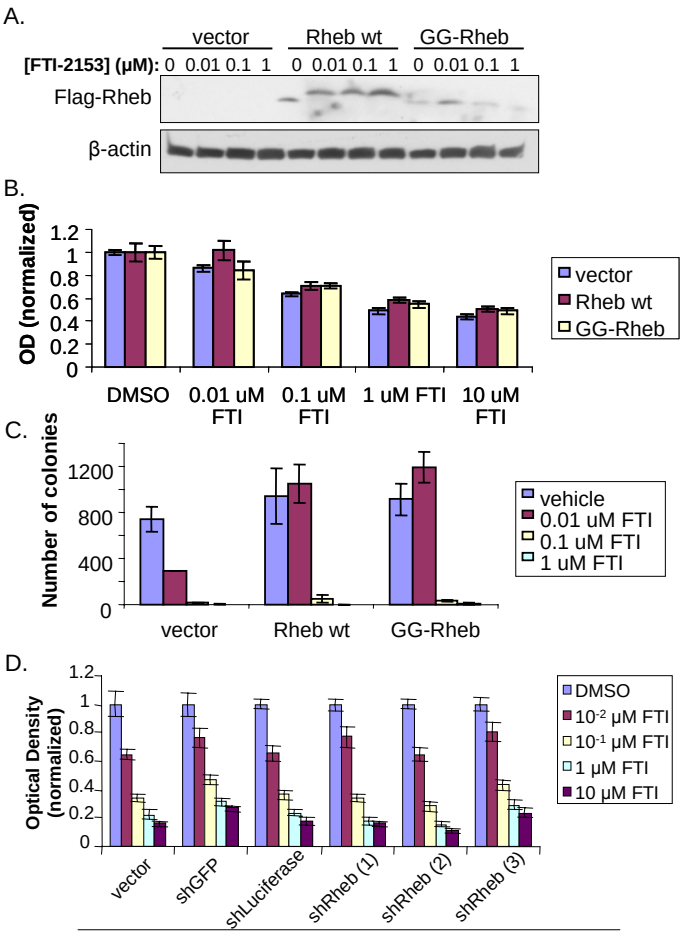

Fig. 4. GG-Rheb1 does not reduce FTI sensitivity of MCF-7 cells. A. MCF-7 cells were stably transfected with the pcDNA3 empty vector or encoding wild-type Rheb1 or GG-Rheb1 (Rheb CVLL). Lysates of cells treated with the indicated concentrations of FTI-2153 were probed with antibodies to Rheb1 and β-actin (loading control). FTI induced a mobility shift in wild-type Rheb1, but not in GG-Rheb1. **B.** MCF-7 stable cell lines were seeded in replicates of 8 in 96-well plates and treated with DMSO or the indicated concentration of FTI-2153 for 3 days. Cell viability was measured using the MTT assay. Data represent an average of 8 replicate wells; error bars represent standard deviation. **C.** MCF-7 stable cells were seeded in triplicate into 0.4% soft agar over a 0.6% bottom layer. After 22 days, viable colonies were stained in 2 mg/mL MTT. ImageJ software was used to quantify number of colonies from images of scanned plates. Results represent the average of triplicate plates; error bars represent standard deviation. **D.** MCF-7 cells were stably infected with pSuper.retro.puro vector and with vector encoding the indicated shRNA. Cells were seeded in replicates of 8 in 96-well plates and treated with DMSO or the indicated concentration of FTI-2153 for 5 days. Data represent an average of 8 replicate wells; error bars represent standard deviation.

KEY RESEARCH ACCOMPLISHMENTS

Research Accomplishments

- Determined that knocking down endogenous Rheb1 expression in MCF-7 cells has no significant effects on estrogen-induced cell proliferation or on anchorage-independent growth (task 2b).
- Assessed the effects of activated Rheb1 on estrogen-dependent proliferation (Task 2f).
- Determined that knocking down Rheb1 expression did not decreases sensitivity to tamoxifen (Task 3).
- Created MCF-7 cell line stably expressing geranylgeranylated Rheb1 (GG-Rheb) mutant.
- Determined that GG-Rheb1 did not rescue FTI inhibition of MCF-7 cell proliferation and anchorage-independent growth.

Training Accomplishments

- Improved the following techniques: soft agar colony formation assay, estrogen-induced cell proliferation/MTT assay, immunofluorescence, and confocal microscopy.
- Attended and presented research at the Department of Defense Breast Cancer Research Program Era of Hope 2008 Meeting.
- Presented poster at the 2008 Annual UNC Genetics & Molecular Biology Annual Retreat.
- Presented research seminar at the Curriculum in Genetics and Molecular Biology Student Seminar Series.
- Presented work in lab meetings and participated in lab journal clubs.
- Mentored rotating graduate student on breast cancer project.

REPORTABLE OUTCOMES

- Stable cell lines developed:
  - MCF-7 cells stably expressing Rheb1 shRNA
  - MCF-7 cells stably expressing activated Rheb1
  - MCF-7 cells stably expressing the FTI-insensitive, functional GG-Rheb1 mutant

- Manuscripts:
  Hanker, A.B., and Der, C.J. (in press). The Roles of Ras Family Small GTPases in Breast Cancer. In Handbook of Cell Signaling, M. Korc, ed. *(review)*

- Abstracts and Presentations:
  UNC Genetics & Molecular Biology Annual Retreat, Sept. 2008
  Poster: Blocking Rheb Posttranslational Processing and Localization

  Department of Defense Breast Cancer Research Program Era of Hope Meeting, June 2008
  Poster: Targeting Rheb Signaling and Posttranslational Processing in Breast Cancer

CONCLUSION

Due to the documented importance of the Rheb-mTOR pathway in cancer [16, 17], and because upstream regulators of the Rheb-mTOR pathway are frequently altered in breast cancer [18, 19], I hypothesized that Rheb1 may be critical for breast cancer progression. Specifically, I focused on the role of Rheb1 in estrogen-induced breast cancer growth. However, I found that knocking down Rheb1 by RNAi did not drastically affect estrogen-induced cell proliferation or anchorage-independent growth. Even though I achieved strong suppression of endogenous Rheb1 protein expression, my results indicated that the Rheb1 pathway was no longer suppressed after long-term estrogen treatment. The Rheb isoform Rheb2 is also expressed in breast cancer and may compensate for the loss of Rheb1. Therefore, it will be necessary to knock down Rheb2 together with Rheb1 in order to remove all Rheb activity and to elucidate the role of Rheb proteins in breast cancer. Since it remains possible that Rheb activity is important in breast cancer, it will be imperative to identify pharmacological agents that inhibit Rheb1 and Rheb2 function for the treatment of breast cancer. One possibility is the class of drugs known as FTIs, which are currently being used to treat breast cancer. However, the molecular targets of these drugs are currently unknown, and elucidating which FTase substrate is responsible for the anti-tumor effects of FTIs will be critical for defining a patient population that will benefit from treatment with FTIs. It has been suggested that Rheb may be a clinically relevant target of FTIs [5, 7, 20, 21]. However, my results suggest that Rheb1 is not the critical FTI target in breast cancer, and instead, other potential targets should be pursued. My future work will focus on identifying other pharmacological agents that block Rheb signaling and Rheb function, and determining whether these agents will be effective therapies for breast cancers with elevated Rheb activity.

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